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Center of Excellence for Therapeutic Individualization for Breast Cancer
Annual Report: General Overview

The report submitted herein includes reports from subcontractors involved in the COE, which explain in detail the efforts of the COE during the past year. This report will highlight the overall progress made by the Center of Excellence.

1. Creation of Research Infrastructure: During the past 12 months, the COE has maintained and expanded the research infrastructure necessary for carrying out the proposed research.
    a. Intra-program communications: We continue to perform regular monthly teleconferences linking the principal investigators, and our face-to-face meetings (at the December 2006 San Antonio Breast Cancer Symposium and at the June 2007 American Society of Clinical Oncology Meetings.
    b. Clinical program development: We continue to identify clinical sites that will participate in our clinical trials and have taken steps to insure that drugs will be available at our planned foreign site in Lima, Peru, which has recently been approved by the DOD and which is now actively accruing patients. We have continued accrual on our first clinical protocol (COE01, the master clinical protocol that represents the centerpiece of the program), which is now open at multiple sites. We have amended COE01 to utilize formalin-fixed, paraffin-embedded tissues (FFPET), as the use of frozen tissues had become a limiting factor in patient accrual to the trial. The advent of novel technologies allowing for the use of FFPET opens new avenues in patient analysis, and has been a major focus in the past year. With the addition of new sites and of the use of FFPET, accrual to COE01 has increased significantly in recent months.
    c. Our second major trial, COE02, has finally cleared the DOD IRB and will open at Indiana University in the very near future. Other sites involved in COE01 are now in the process of initiating COE02.
    d. Following extensive discussions with DOD staff, we developed a new project, COE 05, focused around the utilization of formalin-fixed paraffin-embedded tissues from patients who have died of metastatic breast cancer and who have received monotherapy with a chemotherapeutic agent for their disease. This trial received DOD IRB approval and has initated sample collection and analysis.
    e. Clinical specimen processing: The COE has developed procedures specific to the processing and shipping of clinical specimens from clinical sites to the Pathology Core laboratory. The Pathology Core Laboratory has recently relocated from at the University of Oklahoma to the University of Colorado (with the transfer there of Dr. Ann Thor, core leader). The Pathology Core lab has successfully processed tissues obtained as part of COE 01. Processing of tissues for COE05 is now ongoing.
    f. Research Core Laboratories: A principal focus of the COE's teleconference has involved the prioritization of clinical specimens for research evaluation. A prioritization process has been for both COE01

and COE05. A laboratory procedure manual has been developed for use. The core research laboratories have developed standards for tissue processing, and (as outlined elsewhere) have hired personnel for specimen processing.

As genomic and proteomic technology change, the research core laboratories are in the process of developing novel approaches to investigation of crucial issues. As mentioned in the Leyland-Jones section of this report, we are actively investigating the performance of genomic analyses on paraffin-embedded tissues, which will significantly expand our ability to analyze patient samples, As outlined in the Chang/Baylor/Genomics section, significant progress has been made by the Baylor subcontractor in genomic analyses for docetaxel. This should prove useful in subsequent analyses.

2. Consumer Advocacy core: The Consumer Core has been heavily involved with all of the above, participating regularly in teleconferences, reviewing clinical protocols and procedure manuals, and helping create information packets for patients potentially interested in the protocol. An Advocate Lecture Series was jointly sponsored by the IU/DOD Breast Cancer Center of Excellence and the Research Advocacy Network (RAN). The purpose of the Lecture Series was to inform advocates about 1) the IU/DOD Breast Cancer Centers of Excellence Grant activities 2) the science being used to accomplish the grant goals and 3) the importance of genomics, pharmacogenetics and biospecimen collection and storage in making targeted treatments available to patients. The series included three 1 hour presentations.
3. The Genomics Core (Chang Laboratory) has worked to improve gene expression arrays obtained from small tissue samples, as a technical development. Using tissue obtained through non-DOD studies, the Chang lab identified gene expression patterns that predicted response to neoadjuvant docetaxel.
4. The Pharmacodynamics/Pharmacogenomics core (Leyland-Jones) has established several techniques for use in formalin-fixed, paraffin-embeded tissues, including: 1) FISH (Fluorescent *In Situ* Hybridization) used for the detection of amplification or deletion of several genes including, topoisomerase II A (TOP2A), a well known target of anthracyclines (Arm A), thymidylate synthetase (TS), thymidine phosphorylase (TP) and dihydrofolate reductase (DHFR), these later three are involved in capecitabine metabolism (Arm B). 2) qRT-PCR (quantitative reverse transcriptase-polymerase chain reaction) to measure expression levels of key enzymes involved in the metabolism of capecitabine. In the case of Vinorelbine (Arm C), we have established RT-PCR protocols enabling us to measure mRNA levels of two promising biomarkers, namely β-tubulin III and stathmin from fresh frozen tissue. RT-PCR will also be used to measure tumor expression levels of deoxycytidine kinase (dCK) and ribonucleotide reductase M1 (RRM1), two enzymes involved in the metabolic pathway of gemcitabine. 3) IHC (immunohistochemical) assays, set up in collaboration with Dr. MacKey, will be used to assess levels of two transporters, the human

concentrative nucleoside transporter (hCNT) and the human equilibrative nucleoside transporter 1 (hENT1), both involved in the cellular transport of gemcitabine (Arm D).

In addition, the Leyland-Jones laboratory has developed the Illumina DASL assay for use in FFPET. Using samples collected in non-DOD studies, they have shown here that highly degraded RNA prepared from FFPE breast tumor tissue samples, is amenable to molecular profiling of 502 genes using the DASL assay. The correlation between receptor status, as measured by IHC and FISH, and receptor intensity, as measured in the DASL assay, was remarkably robust. Cluster and heat map analysis (data not shown) of the data was also performed and showed that the samples fell into several groups with receptor status being one but not the only factor at play. Genes were identified whose expression correlated with ER expression, HER2 amplification or triple negative status. Taken together, these data indicate that the DASL assay is an appropriate method to with which to molecularly profile FFPE tumor samples from breast cancer patients.

5. The Proteomics Core (Hickey) has focused its efforts on two issues: a) optimization of biostatistical methodology for SELDI spectral analysis; and b) optimization of techniques for protein extraction from FFPET, both of which are outlined in their report. The switch to FFPET in the COE mandates development of new techniques, both statistical and laboratory, for evaluation of proteomic data.

The Center of Excellence for Therapeutic Individualization for Breast Cancer is posed to analyze significant data sets within the upcoming year, based on our conversion to FFPET tissue sets.

**Summary of work on the DOC BC COE -Hickey lab.**

1) **Optimization of the Biostatistical Methodology for SELDI spectra analysis – Drs. Hickey, Li, and Shen, and L. Dobrolecki.**

Controversy over the most optimal way to analyze SELDI-TOF mass spectral data has been discussed for several years by a variety of authors expressing the pro's and con's for specific individual methods of statistical analysis applied to the raw data accumulated during mass spectral analysis of patient serum samples. This discussion prompted us to develop a specific data set from patient serum samples that could be evaluate by each of 12 different analytical methodologies. Our goal was to identify the methodology giving us the most consistent marker identification between individual patient specimens, with the least amount of variability in marker assignment between duplicates of reach specimen analyzed. The results of this statistical analysis on the 70+ patient samples indicates that the Support Vector Machine with a Gaussian Radial Transformation methodology performs better than the other analytical methods examined. Our results have been written into a manuscript that is now under review by the Journal of Cancer Informatics.

2) **Q-proteome Kit for protein extraction from slides – Hickey**

Because of the limited progress being made in securing IRB approval for the 5 trials proposed for the COE, the decision was made to begin to collect tissue specimens and determine whether sufficient quantities of protein could be obtained from these formalin fixed paraffin embedded specimens to justify performing a SELDI mass spectral analysis on these tissue specimens. The primary problem with these types of specimens is that the fixation process cross-links the proteins within the cells of the various tissue specimens, and significantly reduces the ability of most current methodologies to release these fixed proteins. To overcome this problem, we used a protein extraction kit market by Qiagen, Ltd. to pre-treat the fixed tissue specimens prior to extracting them and utilizing the extracted proteins for SELDI analyses. The pre-treatment is reported to remove the protein cross-links resulting from the formalin fixation. Tissue extracts were then placed on CM-10 (anion ion exhange), and IMAC-Cu (metal ion binding), chips and the chips were subjected to SELDI analysis. Because of the limited quantity of protein material we could obtain from these tissue specimens, we postponed spotting additional extracts prepared from other test slides on H50 (hydrophobic) chips and Q10 chips(cation exchanger). The results of our analyses indicated that the recovery of protein from the formalin fixed tissue specimens was limited, though detectable spectra could be obtained. Initial analyses on the low molecular weight regions of the spectra yielded only several usable peaks that could be reliably seen. There peaks are most likely fairly abundant proteins. The most useful spectral mass range appears to be between 5-20 kDa. The protein yield is approximately 15-30 ug / 2slides. Our next analysis will attempt to increase the quality of the spectra by placing the contents of an entire slide on the surface of one chip type, and subjecting the captured proteins to mass spectral analysis. At this point we cannot say with any degree of certainty that useful information will come from the SELDI analysis of these paraffin embedded tissue specimens.

In parallel with these analyses, we have developed a highly specific antibody (caPCNAab) to a cancer associated isoform of the protein Proliferating Cell Nuclear Antigen. The antibody appears to have the unique ability to selectively identify breast cancer cells, by binding selectively to the caPCNA isoform of PCNA expressed by these cancer cells. Our goal is to validate the clinical efficacy of the caPCNA biomarker and the usefulness of the

caPCNAab as the first diagnostic antibody for breast cancer. In addition, to the IHC studies, I have also developed a robust ELISA assay employing this antibody. The ELISA is selective for caPCNA, and can detect as little as 1 ng of caPCNA/assay well. The analysis of serum specimens (once collected) can potentially identify the presence of circulating caPCNA, that, if found in the serum of an untreated patient, can be used to monitor the effectiveness of treatment and/or the remission status of the patient. Our analysis awaits collection of the serum specimens.

3) **Established a Data Link for transferring SELDI-TOF MS to Biostatistics.**

Working with Greg Peets, in Biostatistics, we developed a secure path to the server hosting the SELDI data we plan to generate during the analysis of these serum specimens. The path represents a secure way to provide Dr. Shen with the raw and processed SELDI data we expect to generate during the proteomic studies and that he needs for his data analyses.

**Subject: Annual Report for Genomics Core**

**Introduction**

Optimal systemic treatment after breast cancer is the most crucial factor in reducing mortality in women with breast cancer. Adjuvant chemotherapy and hormonal treatment both reduce the risk of death in breast cancer patients. However, while estrogen receptors status predicts for response to hormone treatments, there are no clinically useful predictive markers for chemotherapy responses. All eligible women are therefore treated in the same manner. Even denoval drug resistance will result in treatment failures in many breast cancer patients. Currently, there are no methods available to distinguish those patients who are likely to respond to specific chemotherapies, and given the accepted practice of prescribing adjuvant treatment to most parties, even if the average expected benefit is slow, the selection of appropriate patients represents a major advance in the clinical management of breast cancer today.

We therefore set out to identify gene expression patterns in breast cancer specimens that might predict response to taxenes. Chemotherapy allows for the sampling of the primary tumor for gene expression analysis and for direct assessment of response to chemotherapy by following changes in tumor size during the first few months of treatment. Hence, chemotherapy provides an idea platform to rapidly discover predictive markers of chemotherapy response.

In this present study, we hypothesize through high quantitation of gene expression, grade is possible to access thousands of genes simultaneously, and expression patterns in different breast cancers might correlate with and thereby predict response to treatment. The purpose of this study was to (1) demonstrate that sufficient RNA could be obtained from core biopsies to access gene expression, (2) to identify groups of genes that could be used to distinguish primary breast cancers to responsive or resistance to different chemotherapies, and (3) to identify gene pathways that could be important in a mechanism of action of these agents. Most of the gene expression experiments have been based on fresh frozen or recently fixed material. We have extended on these technologies to include the use of formalin-fixed, paraffin-embedded (FFPE) material for high throughput genomic analysis. This would enable easier access to tissue repositories that may enable us to discover predictive genes for therapy response.

**Body of Research**

We have been improving gene expression arrays obtained from small tissue samples, as technical development. We measured each core biopsy obtained from primary breast cancers as approximately 1 cm x 1 mm. As these core biopsies were too small for micro dissection, we ascertained the tumor cellularity of the pretreatment core biopsies. In general, the core biopsies showed good tumor cellularity with median tumor cellularity of 75% (range 40-100%). Each core biopsy yielded 3-6 mg of total RNA, which is more than sufficient to generate approximately 20 mg of label cRNA needed for hybridization with the Affymetrix U133A Genechip, using the manufacturer's standard protocols. We have also experimented with laser microdissection of tumors of lower tumor cellularity.

Previously, we had identified gene expression patterns that predicted response to neoadjuvant docetaxel. Other studies have validated that a high Recurrence Score (RS) by the 21-gene RT-PCR assay is predictive of worse prognosis but better response to chemotherapy. We investigated whether tumor expression of these 21 genes and other candidate genes can predict response to docetaxel. Core biopsies from 97 patients were obtained before treatment with neoadjuvant docetaxel (4 cycles, 100 mg/m2 q3 weeks). Three 10-micron FFPE sections were submitted for quantitative RT-PCR assays of 192 genes that were selected from our previous work and the literature. Of the 97 patients, 81 (84%) had sufficient invasive cancer, 80 (82%) had sufficient RNA for QRTPCR assay, and 72 (74%) had clinical response data. Mean age was 48.5 years, and the median tumor size was 6 cm. Clinical complete responses (CR) were observed in 12 (17%), partial responses in 41 (57%), stable disease in 17 (24%), and progressive disease in 2 patients (3%). A significant relationship ($p<0.05$) between gene expression and CR was observed for 14 genes, including CYBA. CR was associated with lower expression of the ER gene group and higher expression of the proliferation gene group from the 21 gene assay. Of note, CR was more likely with a high RS ($p=0.008$). We have established molecular profiles of sensitivity to docetaxel. RT-PCR technology provides a potential platform for a predictive test of chemosensitivity using small amounts of routinely processed material.

High throughput genomics offers a potential predictive test of sensitivity for different treatments, and may thereby allow selection of the most appropriate therapy for breast cancer patients, as we propose here.

References:


1. **J Chang**, A Makris, M Gutierrez, S Hilsenbeck, J Hackett, J Jeong, ML Liu, J Baker, K Clark-Langone, F Baehner, K Sexton, S Mohsin, T Gray, L Alvarez, G Chamness, C Osborne, S Shak. Gene expression patterns in formalin-fixed, paraffin-embedded core biopsies predict docetaxel chemosensitivity in breast cancer patients. *Breast Cancer Research and Treatment*, 2007 (in press)
2. **JC Chang**, A Makris, SG Hilsenbeck, JR Hackett, J Jeong, M Liu, J Baker, K Sexton, CK Osborne, S Shak. Gene expression profiles in formalin-fixed, paraffin-embedded (FFPE) core biopsies predict docetaxel chemosensitivity. Proceedings of the American Society of Clinical Oncology (ASCO) 42nd Annual Meeting 2006.
3. **JC Chang**, Xiaoxian Li, J Rosen, W Bu, H Wong, X Zhang, L Moreno, H Weiss, A Tsimelzon, , SG Hilsenbeck, AV Lee, CK Osborne, G Dontu, M Wicha and MT Lewis. Breast cancer stem cells are responsible for therapeutic resistance and residual disease. San Antonio Breast Cancer Symposium 2006.

# Hoosier Oncology Group Subcontract Report

**Statement of Work Task 1: Development of/Preparation for Metastatic Chemotherapy Parent Protocol**
5 sites (4 US and 1 Peru) open under the Master Protocol dated 20MAY2005

5 additional US sites are in the regulatory process for opening under the Protocol Amendment 12JAN2007. This amendment will allow patients to enroll to the trial without obtaining fresh frozen tissue as long as a formalin-fixed paraffin embedded tissue block is available for analysis.

All sites will submit this amendment to assist in patient enrollment on this trial. Of the 5 current open sites, only one has the amended protocol approved and is awaiting the Department of Defense's approval for enrollment of patients. All remaining sites are in the process of obtaining approval of the amended protocol.

Monthly teleconferences conducted with George Sledge, M D and Cores Face-to-Face meetings conducted 03JUN2006 and 15DEC2006.

The second protocol is in the final review process for one site. This site is expected to open for enrollment in May 2007. The database and sample collection will be completed in April 2007.

There are approximately 7 sites working to complete all regulatory documents for participation in this trial

The third protocol draft and informed consent draft are completed. The company has reviewed these documents and given their approval to move forward. Expected start of this trial is January 2008

There is a Retrospective / Prospective Research Plan currently collecting data and formalin-fixed paraffin embedded tissue samples. 20 samples have been retrieved to date.

**Statement of Work Task 2: Performance of Metastatic Chemotherapy Trial/Tissue Collection/Patient Follow-up**
Three patients enrolled to the trial in the US. Tissue collection and submission to central laboratory conducted as planned.
First patient enrolled at Peru site on 30MAR2007. Tissue collection and submission to central laboratory conducted as planned.

**Statement of Work Task 3: Analysis of Tissues by Laboratory Cores**
Awaiting additional patient accrual

**Statement of Work Task 4: Performance of Prospective Validation Trial**
**Statement of Work Task 5: Performance of Investigational Agent Trials**
Awaiting completion of Task 1-3

# VMI Institute Subcontract Report

- Pharmacodynamics/Pharmacogenomics Core

  Dr. Brian Leyland-Jones

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## *Introduction*

The primary objective of the pharmacodynamics/pharmacogenomics core facility is to develop user-friendly techniques readily available to the clinician for measuring a specific aspect of response and/or toxicity, which will lead to the individualization of therapy. Critical determinants that govern individual responsiveness will be identified. These include markers and kinetic rate or metabolic outcome, which are often referred to as a pharmacokinetic or pharmacodynamic "signature". One critical advantage of measuring these "signatures" is that they will be directly compared to and contrasted with the genomic and proteomic analyses.

In order to identify such pharmacokinetic signatures, a number of techniques have been established in our lab that include: 1) FISH (Fluorescent *In Situ* Hybridization) used for the detection of amplification or deletion of several genes including, topoisomerase II A (TOP2A), a well known target of anthracyclines (Arm A), thymidylate synthetase (TS), thymidine phosphorylase (TP) and dihydrofolate reductase (DHFR), these later three are involved in capecitabine metabolism (Arm B). 2) qRT-PCR (quantitative reverse transcriptase-polymerase chain reaction) to measure expression levels of key enzymes involved in the metabolism of capecitabine. In the case of Vinorelbine (Arm C), we have established RT-PCR protocols enabling us to measure mRNA levels of two promising biomarkers, namely β-tubulin III and stathmin from fresh frozen tissue. RT-PCR will also be used to measure tumor expression levels of deoxycytidine kinase (dCK) and ribonucleotide reductase M1 (RRM1), two enzymes involved in the metabolic pathway of gemcitabine. 3) IHC (immunohistochemical) assays, set up in collaboration with Dr. MacKey, will be used to assess levels of two transporters, the human concentrative nucleoside transporter (hCNT) and the human equilibrative nucleoside transporter 1 (hENT1), both involved in the cellular transport of gemcitabine (Arm D).

Assays already validated on fresh frozen tissue, were revalidated on a set of formalin-fixed paraffin-embedded (FFPE) tumor tissue coming from five different patients. This will allow the use of such archival specimens and increase the number of observations for each treatment arm.

Correlation of the protein/enzyme activity profile with disease state, therapy and drug response will provide invaluable insight into monitoring inter-individual variations in efficacy and toxicity. Moreover, these observations could be used to help select appropriate drug and dosage regimens for each patient.

## *Validation of Assays:*

### Arm A: Doxorubicin and Cyclophosphamide

The enzyme Topoisomerase II A (TOP2A), which catalyses the breakage and reunion of double-stranded DNA, plays an important role in a number of fundamental nuclear processes including DNA transcription, replication and recombination. In addition, topoisomerases are required for maintaining proper chromosome structure and segregation[1]. Type II topoisomerases are targets for the anthracycline class of anticancer drugs, such as doxorubicin and epirubicin, which are also termed topoisomerase inhibitors[2].

The TOP2A FISH assay is used to determine the copy number of the TOP2A gene, which is located on chromosome 17q21-q22, using the chromosome 17 centromere region (Cen17) as a reference[3]. Two copies of the TOP2A gene are present in all normal diploid cells and in some carcinomas, whereas, the TOP2A/Cen17 ratio is higher in cells when the TOP2A gene is amplified (defined as $\geq 2$) or lower in cells when the TOP2A gene is deleted (defined as $< 0.8$).

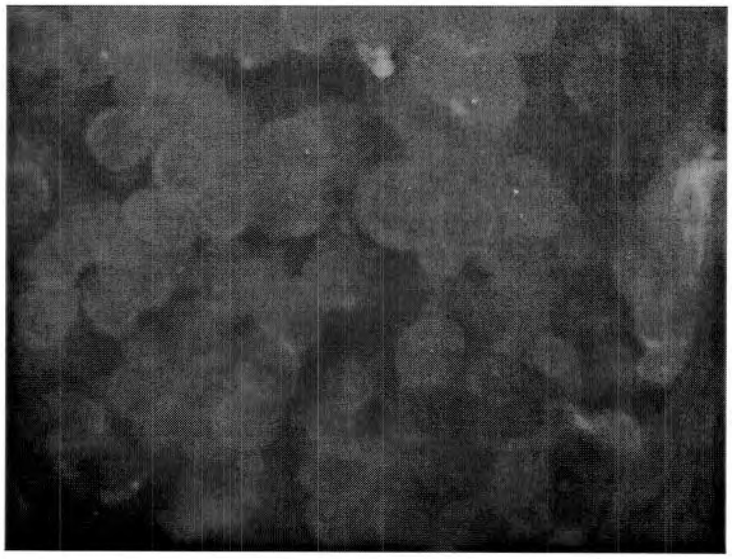

Figure 1: Representative photograph of cells having HER2 gene amplification, with two signals for the centromere of chromosome 17 (green dots) and multiple signals for HER2 (red dots) per nucleus (blue). It should be noted that in the photographs presented in figures 1 and 2, only one plane of focus is shown, whereas the scoring of green and red dots used to calculate the ratio was performed in all focus planes. 1000X magnification (immersion).

Results for the FFPE validation set of five patients are shown in table 1. Only one patient out of five had HER2 amplification (HER2/Cen17 ≥ 2) along with TOP2A gene deletion (TOP2A/Cen17 < 0.8).

Table 1: Alteration of gene copy number for HER2 and TOP2A assessed using FISH

| Patient | HER2/CEN ratio | HER2 status | TOP2A/CEN ratio | TOP2A status |
|---|---|---|---|---|
| TRN-0002 | >5 | Amplified | 0.63 | Deleted |
| TRN-0003 | 0.91 | Normal | 0.86 | Normal |
| TRN-0004 | 0.92 | Normal | 1.30 | Normal |
| TRN-0010 | 1.06 | Normal | 0.87 | Normal |
| TRN-0013 | 0.93 | Normal | 1.04 | Normal |

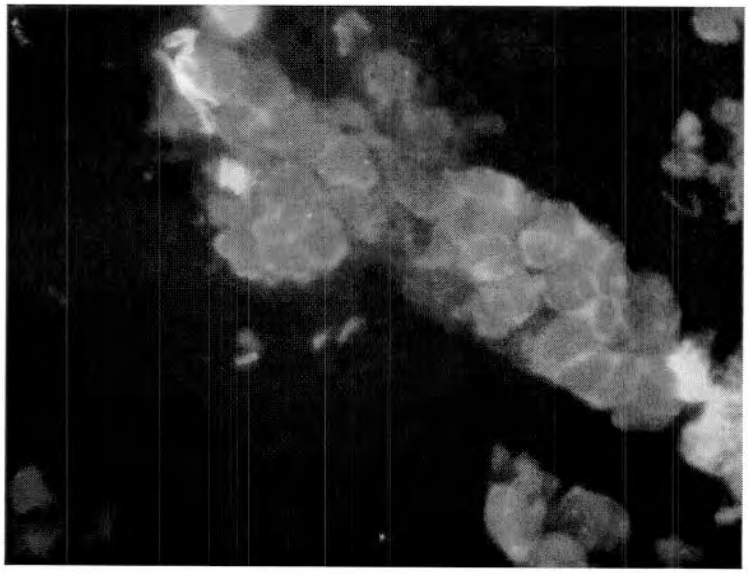

Figure 2: Representative photograph of cells having TOP2A gene deletion, with two signals for the centromere of chromosome 17 (green dots) and less than two signals for HER2 (red dots) per nucleus (blue). 1000X magnification (immersion).

## Arm B: Capecitabine

Fluoropyrimidines, with 5-fluorouracil (5-FU) as one of its principal representatives of this class of chemotherapeutic agents, have been the standard treatment for a wide range of common solid tumors including breast cancer. Attempts to increase the efficacy and tolerability of fluoropyrimidine treatment have led to the development of capecitibine, a fluoropyrimidine prodrug, which is preferentially activated at the tumor site by the enzyme thymidine phosphorylase (TP)[4]. Capecitabine and its intermediate metabolites, 5'deoxy-5-fluorocytidine (5'-DFCR) and 5'-deoxy-5-fluorouridine (5'-DFUR), are not cytotoxic but become effective only after they have been converted to 5-FU by TP[5]. One of the principal mechanisms of 5-FU action is inhibition of the enzyme thymidilate synthase (TS) by fluorodeoxyuridine

monophosphate (FdUMP), one of several 5-FU metabolites. TS is an important enzyme in pyrimidine metabolism which is crucial for *de novo* synthesis of thymidine nucleotides. Another enzyme, that most likely plays a crucial role in the antitumor activity of 5-FU is dehydropyrimidine dehydrogenase (DPD), because DPD is responsible for the catabolic conversion of 5-FU to an inactive metabolite and decreases 5-FU levels within cells[6,7]. Therefore, the fluoropyrimidine pathway enzymes, TP, TS, DPD and DHFR, may be potential candidate biomarkers that could be used to predict tumor response to capecitabine.

For investigation of TS, DHFR and TP gene copy number, newly developed FISH probes (Dako, Glostrup, Denmark) have been used on 5µm FFPE tissue slices. Hybridization signals have been evaluated using the ratio of red signals for TS, DHFR or TP to green signals for the centromere of the relevant chromosome (CEN) in at least 20 morphologically intact and non-overlapping nuclei. Tumors have been classified as TS, DHFR or TP amplified (TS/CEN, DHFR/CEN or TP/CEN ≥ 2.0), or deleted (TS/CEN, DHFR/CEN or TP /CEN ≤0.8). Gene copy number alterations for TS, DHFR and TP in the FFPE validation set are shown in table 2.

Table 2: Alteration of gene copy number for TS, DHFR and TP assessed using FISH

| Patient | TS/CEN | TS | DHFR/CEN | DHFR | TP/CEN | TP |
|---|---|---|---|---|---|---|
| TRN-0002 | 1.2 | Normal | 1.8 | Normal | 1.2 | Normal |
| TRN-0003 | 0.9 | Normal | 1.2 | Normal | 1.2 | Normal |
| TRN-0004 | 0.9 | Normal | 1.1 | Normal | 1.0 | Normal |
| TRN-0010 | 1.3 | Normal | 1.6 | Normal | 2.0 | Normal |
| TRN-0013 | 1.8 | Normal | 1.3 | Normal | 1.5 | Normal |

Quantitative assessment of gene expression by RT-PCR assays enables the use of limited samples of fresh frozen tissue as well as of formalin fixed

paraffin-embedded (FFPE) tissue in a very short time. Quantitative analysis of DPD, TP or TS mRNA levels is conducted by a two-step procedure. In the first step, cDNA is reverse transcribed from total RNA using AMV reverse transcriptase and random hexamer priming. In the second step, a fragment of DPD, TP or TS-encoding mRNA is amplified from the cDNA by PCR using gene specific primers. The amplicon is detected by fluorescence using a specific pair of hybridization probes[8]. In the same cDNA preparation but in a separate PCR reaction, mRNA encoding for glucose-6-phosphate dehydrogenase (G6PDH) is quantified for use as a reference (housekeeping) gene. The DPD, TP or TS expression level is quantified relative to G6PDH and a calibrator RNA, eliminating the need of a standard curve.

Expression of TS, DPD and TP was assessed on the FFPE validation set and the data is presented in table 3.

Table 3: Gene expression of TS, DPD and TP using RT-PCR expressed as Ct values (point at which the fluorescence crosses the threshold).

| Patient | TS | DPD | TP | G6PDH |
|---|---|---|---|---|
| TRN-0002 | 24 | 31 | 32 | 29 |
| TRN-0003 | 24 | 35 | 24 | 29 |
| TRN-0004 | 27 | 33 | 26 | 24 |
| TRN-0010 | 31 | 35 | 30 | 34 |
| TRN-0013 | 32 | 34 | 23 | 34 |

## Arm C: Vinorelbine

Antitubulin agents such as taxanes and vinorelbine are widely used in the treatment of patients with breast cancer. The target of these compounds, microtubules, are complex polymers consisting of α/β tubulin heterodimers and a variety of microtubule-associated proteins to which these compounds

bind. The expression of certain tubulin isotypes have often been found to be correlated with their sensitivity to antitubulin agents[9]. Recently, Rosell *et al* used quantitative RT-PCR to analyze the expression of β-tubulin III and stathmin mRNA isolated from FFPE tumor biopsies from NSCLC patients treated with vinorelbine and showed that time to progression was influenced by β-tubulin III and stathmin levels[10].

Expression of β-tubulin III and stathmin was assessed on the FFPE validation set of five patients (table 4).

Table 4: Gene expression of β-tubulin III and stathmin using RT-PCR expressed Ct values (point at which the fluorescence crosses the threshold).

| Patient | β-tubulin III | stathmin | G6PDH |
|---|---|---|---|
| TRN-0002 | 24 | 31 | 27 |
| TRN-0003 | 36 | 42 | 24 |
| TRN-0004 | 38 | 29 | 27 |
| TRN-0010 | 29 | 41 | 32 |
| TRN-0013 | 34 | 40 | 33 |

## Arm D: Gemcitabine

Activation of gemcitabine requires phosphorylation to mono-, di-, and triphosphates. Similar to the structurally and functionally related deoxycytidine analog ara-C, the first crucial step is phosphorylation catalyzed by deoxycytidine kinase (dCK)[11]. However, in contrast to ara-C, gemcitabine has multiple intracellular targets; up- or down-regulation of these targets may confer resistance to this drug. Recent studies show a strong correlation between sensitivity to gemcitabine as well as the most important metabolite of gemcitabine, dFdCTP and dCK activity. A potential important role could also be played by ribonucleotide reductase (RRM1), one of gemcitabine's targets, along with members of specialized transport systems required for the

passage of nucleoside analogs. dCK and RRM1 may be potential candidate biomarkers that could be used to predict tumor response to gemcitabine.

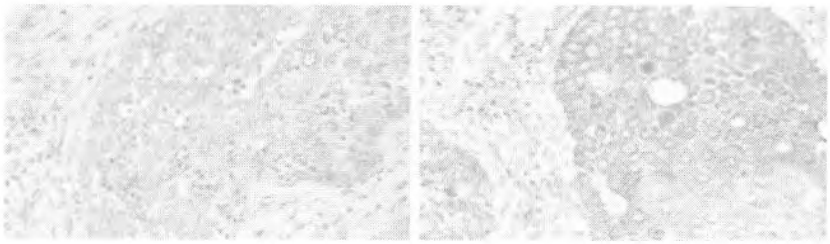

Figure 3: Immunocytochemical staining of deoxycytidine kinase (dCK). Phosphate buffered saline negative control (left) and sample with high dCK expression (right).

## *RNA Yield and Quality from FFPE Breast Tumor Samples:*

We have performed gene expression profiling of FFPE breast tumors using the DASL assay. In his study, all breast tumor samples were invasive ductal carcinomas (IDC) that had been taken within the last two years (2004-2005) and for which detailed patient information was available. Total RNA was extracted from three 5 µm thick FFPE sections (tumor blocks were at least 1 $cm^2$) containing at least 80% IDC cells using the High Pure RNA Paraffin Kit according to the manufacturer's instructions (Roche Diagnostics; Indianapolis, IN). Total RNA yields ranged from 0.64 to 14.1 µg, and in most cases, at least 1 µg from each patient's sample with an average yield of greater than 3 µg of total RNA was obtained (data not shown). The RNA is highly degraded due to the formalin fixation process used to preserve tumor tissue. The size of the RNA fragments purified from FFPE sections ranged from 50 to 700 nucleotides, with an average size of ~175. In addition, these size fragments are amenable to RT-PCR techniques, which use probe sets that can result in the amplification of relatively small products in the 50 to 100 base pair range.

Therefore, techniques that employ RT-PCR-based strategies, such as the DASL assay, can utilize RNA prepared from FFPE sections.

To test the quality of the extracted RNA, qRT-PCR was performed for the highly abundant transcript of the ribosomal protein L13a (RPL13a). When qRT-PCR is performed on RNA prepared from FFPE sections using the RPL13a probe set, the $C_t$ value expected should be not be more that 12 cycles greater when compared with control RNA. In general, the $C_t$ values (range 26-34) were at the high end of the QC range and from 10 to 17 cycles more (data not shown). In most cases, when the QC assay was repeated using more input RNA, the $C_t$ values obtained were now within the acceptable range. Therefore, depending on the results from the QC assay, the amount of input RNA used in the DASL assay ranged from 200 to 500 ng. Results from experiments conducted at Illumina demonstrated that input RNAs with $C_t$ value of 28 cycles or less can be used successfully in the DASL assay. .

## *DASL Assay Analysis of Invasive Ductal Carcinoma Tumors from 50 patients:*

RNA was prepared from 50 FFPE IDC tumor samples, with different receptor status as shown below and analyzed in the DASL assay using the 502-gene cancer panel from Illumina.

ER+
- ER+, PR+, HER2 ----------13
- ER+, PR-, HER2 ---------- 7

HER2+
- HER2+, ER+, PR±----------12
- HER2+, ER-, PR---------- 9

Triple negative
- ER-, PR-, HER2- ---------- 9

Total = 50

Unsupervised hierarchical clustering of the samples was performed and is shown in Figure 4. All replicate samples clustered together as expected. Samples clustered into several groups reflecting tumor heterogeneity.

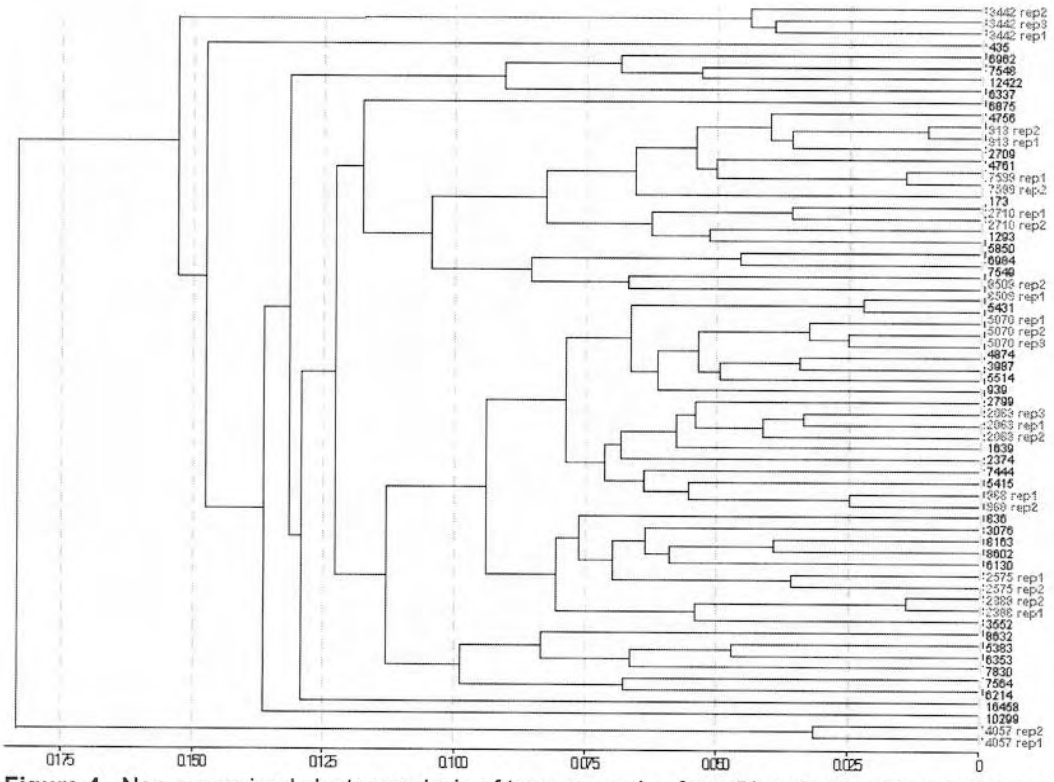


**Figure 4.** Non-supervised cluster analysis of tumor samples from 50 patient's with invasive breast cancer using Illumina's 502-gene human cancer panel and analyzed on a 96-Sentrix Array Matrix. All 10 replicate samples (shown in red) cluster together.

## Comparison between DASL and HER2 IHC/FISH data and between DASL and ER and PR IHC data:

To determine whether the DASL assay yields comparable data to IHC and FISH analyses, the DASL assay gene intensity (expression) data was compared with the available expression data for HER2, ER, and PR on the set of tumors from the 50 patients. The comparison of data sets for HER2, ER, and PR are shown in **Figures 5, 6, and 7,** respectively (for graphing purposes, HER2 FISH data was scored as positive = 100 and negative = 0

and for ER and PGR IHC data, negative = 0, weakly positive (+) = 34, moderately positive (++) = 67, and strongly positive (+++) = 100).

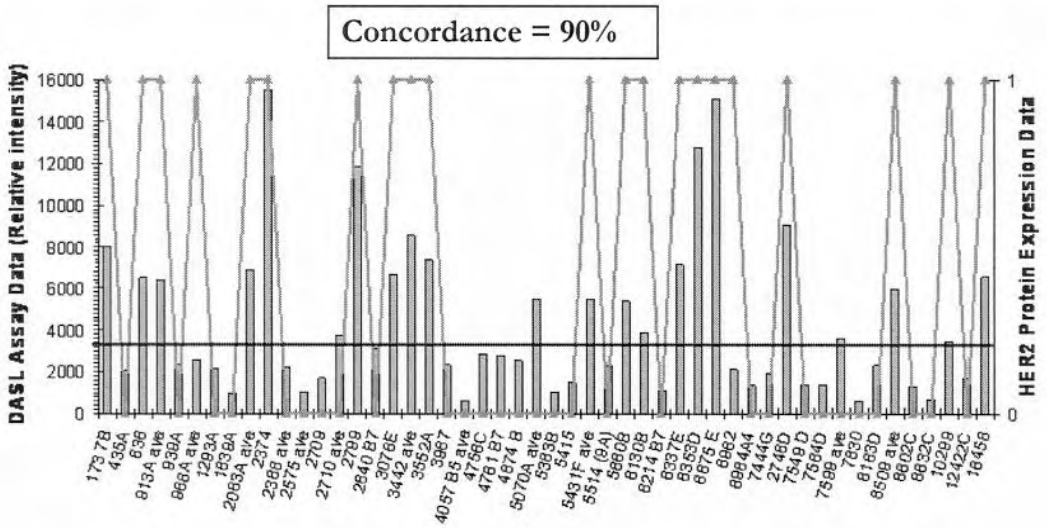


**Figure 5.** Comparison of DASL data and IHC/FISH data for HER2 in 50 cases of IDC. DASL assay data shown in the blue bars, IHC/FISH data shown in orange. For HER2 data, non-amplified = 0 and amplified = 100. Black bar across each graph indicates cutoff (sensitivity/specificity) used to determine concordance between assays.

FISH data for HER2 was taken as the validated assay and used to determine sensitivity and specificity for the DASL assay data. In this manner, an intensity level cutoff of 3,200 in the DASL assay was selected (Figure 5). Overall, the concordance between the DASL and FISH assays was 90%. In a similar comparison of HER2 gene expression, measured by qRT-PCR and FISH, only 77% concordance was observed between assays for 43 tumors **(Vinatzer U, Dampier B, Streubel B, Pacher M, Seewald MJ, Stratowa C, Kaserer K, Schreiber M: Expression of HER2 and the coamplified genes GRB7 and MLN64 in human breast cancer: quantitative real-time reverse transcription-PCR as a diagnostic alternative to immunohistochemistry and fluorescence in situ hybridization. Clin Cancer Res 2005; 11(23): 8348-57)**. Therefore, DASL data is at least comparable to qRT-PCR. Data generated in the DASL assay for HER2 could be potentially used to investigate whether or not there is any correlation between DASL intensity, gene amplification score, and clinical outcome. Unfortunately, FISH results

were only reported as being positive or negative (cut-off of two) and no amplification score was given that could be compared with DASL assay intensity data.

The comparison of data sets for ERα is shown in **Figure 6**. Although IHC data in general is more variable and strict comparisons between RNA and protein expression data may not be straightforward, overall, the concordance between the two assays was very high at 88%.

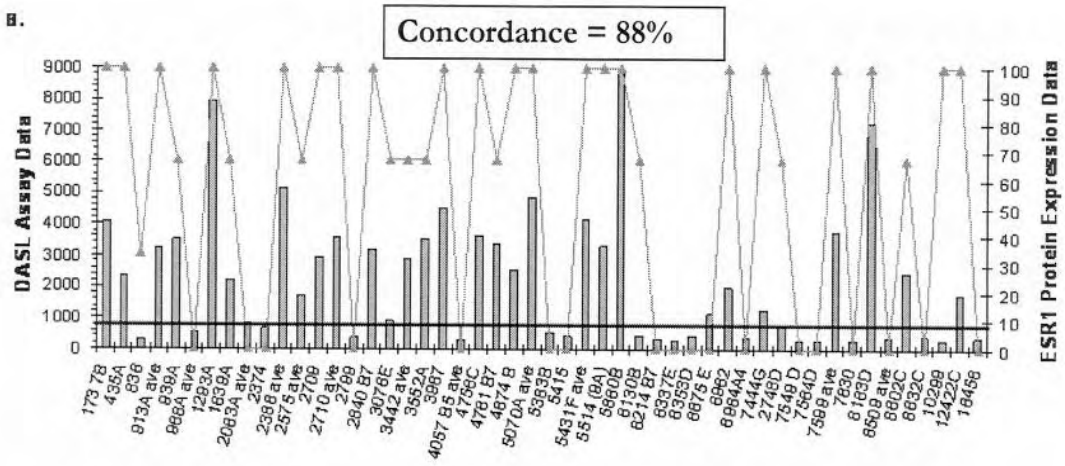


**Figure 6.** Comparison of DASL data and IHC/FISH data for ER in 50 cases of IDC. DASL assay data shown in the blue bars, IHC/FISH data shown in orange. For ESR1 (ER), no staining = 0, + staining = 34, ++ staining = 67, and +++ staining = 100. Black bar across each graph indicates cutoff (sensitivity/specificity) used to determine concordance between assays.

The comparison of data sets for PGR is shown in **Figure 7**. Overall, the concordance between the two assays was very good, at 86%.

We then compared DASL data for ER, PR and HER2 according to receptor group. We show that ER is expressed in the ER-positive tumors and not in the ER negative tumors. Similarly, PR is only expressed in the PR positive tumors. HER2 expression is detected in the HER2 non-amplified tumors but expression increased greatly in the HER2 amplified tumors. Interestingly, we

found that HER2 expression was greater in the ER negative, HER2 positive tumors then in ER positive, HER2 postivie tumor samples. Activated ER been shown to down-regulate expression of HER2 in human breast cancer cell lines (Frasor et al, Endocrinology. 2003 144:4562, 2003). In the TN tumors, virtually no expression of the three receptors was detected. .

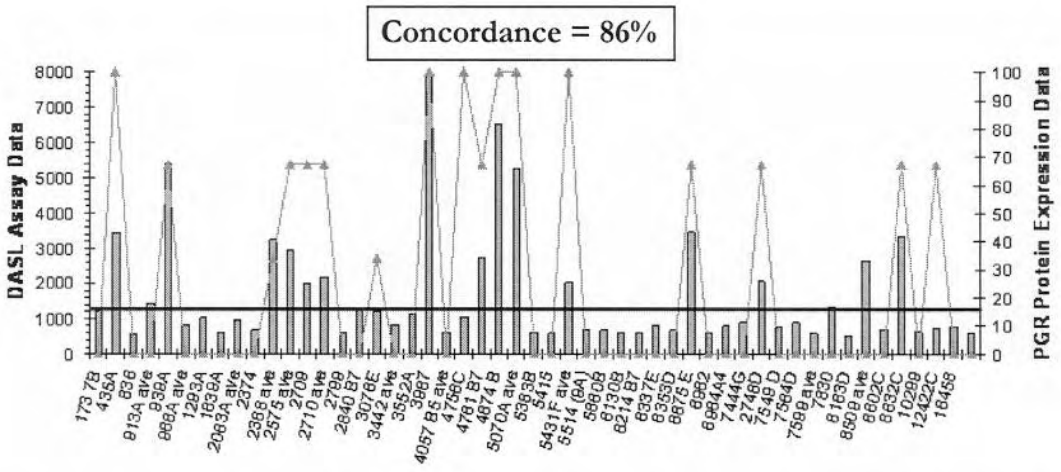


**Figure 7.** Comparison of DASL data and IHC/FISH data for PR in 50 cases of IDC. DASL assay data shown in the blue bars, IHC/FISH data shown in orange. For PGR (PR), no staining = 0, + staining = 34, ++ staining = 67, and +++ staining = 100. Black bar across each graph indicates cutoff (sensitivity/specificity) used to determine concordance between assays.

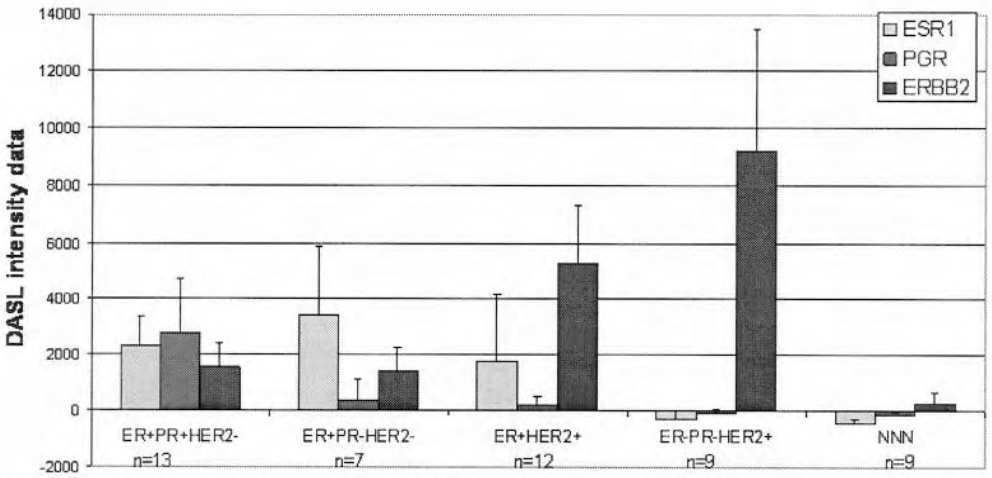


**Figure 8.** Comparison of DASL data for ER, PR and HER2 according to receptor group.

In Summary, total RNA was prepared from 50 FFPE ductal breast cancer tumors with different hormone (ER and PR) and growth factor (HER2) receptor profiles. Molecular profiling using the DASL assay with a 502 cancer-related gene panel from Illumina was conducted. Samples clustered into several groups, with duplicate and triplicate samples showing excellent reproducibility and clustered together as seen in the dendogram. Concordance between the IHC/FISH assays and the DASL assay for HER2, ER, and PGR was 90%, 88% and 86%, respectively. A number of additional expected and unexpected genes were identified in the DASL assay as differentially expressed in the different receptor groups.

In conclusion, we have shown here that highly degraded RNA prepared from FFPE breast tumor tissue samples, is amenable to molecular profiling of 502 genes using the DASL assay. The correlation between receptor status, as measured by IHC and FISH, and receptor intensity, as measured in the DASL assay, was remarkably robust. Cluster and heat map analysis (data not shown) of the data was also performed and showed that the samples fell into several groups with receptor status being one but not the only factor at play. Genes were identified whose expression correlated with ER expression, HER2 amplification or triple negative status. Taken together, these data indicate that the DASL assay is an appropriate method to with which to molecularly profile FFPE tumor samples from breast cancer patients.

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# University of Colorado Subcontract Report

Ann D. Thor

Received and catalogued two new patients on protocol COE-01 and received the end of treatment specimens from a patient from the previous report. Since the response to the request for fresh frozen specimens has limited the availability of patients to this protocol, the PI and core PI's decided to test a new paradigm using formalin fixed paraffin embedded (FFPE) tissue sections We responsed to the PI's request for FFPE tissues from previously diagnosed breast cancer patients by identifying 5 cases from our archival files. After obtaining the specifics from the three core PI's regarding the cutting schema, we provided tissue sections to each core laboratory for testing. We have also reviewed the protocols for COE-02 and COE-03. We are currently awaiting final information form the core PI's regarding a cutting schema for using FFPE specimens from newly acquired patients as well as archival specimens. Both the PI and supervisor were at the June face to face meeting and the supervisor was at the December face to face meeting.

# Research Advocacy Network Subcontract Report

Mary Lou Smith
Elda Railey

# Abstract

Advocate participation in this research study will allow them to better understand the science of genomics, the methodology and the results so they can take the lead in informing the advocacy community about this important project. The patient advocates will create and maintain a network of advocates and advocate organizations.

The Patient Advocates will work closely with the Clinical Trial Core to develop patient education materials and expand recruitment strategies. Patient education materials will be developed in English and some will be available in Spanish.

An educational program for local advocates will increase their awareness and understanding of the science underlying the Center's activities. A Mentor Program for Research Advocates will provide ongoing training to equip advocates to support the goals and objectives of the Center of Excellence. The Mentor Program will include didactic and experiential learnings in genomics, proteomics and pharmacogenetics. Mentors will be chosen from the advocate organizations in Indiana.

To date:

- Lecture Series for Advocates was offered to advocates in March 2006 and archived for ongoing learning
- Patient booklets "Why you should consider donating your tissue for research" were provided in English and Spanish
- IRB booklets "The Importance of Tissue Research" were provided in English and Spanish
- Genomics Workbook for Advocates supplied as the textbook for the Mentor Program
- Planned Mentor Program for Research Advocates – May, 2007

**Center of Excellence for Individualization in Breast Cancer Therapy**
**Advocate Core**

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## Body

During this reporting period, advocates in the Advocate Core participated with researchers to provide the patient perspective to all discussions. Several trips to Indianapolis enabled advocates to meet and interact with the PI and other associates. The meetings revolved around how to effectively educate advocates about genomics, proteomics and pharmacogenetics through didactic and laboratory work using multi-modality approaches. This led to the development of the Mentor Program for Research Advocates planned for 2007.

Advocates worked closely with the PIs to provide appropriate patient and advocate education materials. Advocates participated in problem-solving and clinical trial planning at two face-to-face meetings during the year. One of the meetings was in connection with the ASCO annual meeting and the other took place during the San Antonio Breast Cancer Symposium. An advocate core representative attended the DOD meeting in July and presented the accomplishments of the Advocate Core at the DOD site visit November 2006 in Indianapolis.

An Advocate Lecture Series was jointly sponsored by the IU/DOD Breast Cancer Center of Excellence and the Research Advocacy Network (RAN). The purpose of the Lecture Series was to inform advocates about 1) the IU/DOD Breast Cancer Centers of Excellence Grant activities 2) the science being used to accomplish the grant goals and 3) the importance of genomics, pharmacogenetics and biospecimen collection and storage in making targeted treatments available to patients. The series included three 1 hour presentations. The first presentation was by George W. Sledge, Jr. MD. He provided an overview of the IU/DOD grant, the "omics" involved in the research, the desired outcomes and how advocates can support this research. The second presentation of the series was given by Jenny Chang, MD. Her presentation focused on genomics and cancer and David Flockhart, MD, PhD provided an overview of pharmacogenetics. The third presentation was by Ann Thor, MD discussing the operational issues in collecting and storing biospecimens, especially in multi-centered, multi-country research. The sessions were archived for later playback for advocates unable to attend the live session. Over 60 advocates registered for each of the three programs.

The Mentor Program for Research Advocates was planned during the grant year and will begin on April 1, 2007. Please see the Overview for more information on the planned activities and timeline.

## Overview

***Purpose of the Mentor Program for Research Advocates***

The purpose of the Mentor Program for Research Advocates is to equip experienced advocates to mentor people interested in working with the research community and the Center of Excellence. The content will be focused on providing an understanding of the new science in genomics, proteomics and pharmacogenetics and how this new science applies to research advocacy. Mentor program graduates will be asked to identify another advocate in their organization to mentor to a higher level of involvement in research advocacy for breast cancer.

***Formal Educational Program***

The formal educational program will include both didactic and laboratory work. Experiential learning opportunities will be provided to reinforce and apply the knowledge acquired in the lectures. The formal educational program will be focused on genomics, proteomics and pharmacogenetics. The laboratory work will include opportunities to spin down a sample and follow tissue from sample acquisition to slide preparation and use. Participants will learn about DNA, RNA, microarrays, tissue banks, and mouse models. Participants will learn how samples are stored and existing and proposed standards for tissue banking.

The Program will consist of:

| | |
|---|---|
| Week of April 2, 2007 | **Genomics Lecture Series Begins**<br>Text: Research Advocate Network *Genomics in Cancer: Workbook for Advocates* (print copies will be included for all participants)<br>**Chapter 1** *Genomics: Studying the Bird Instead of the Wing* – Overview of genomics including how it is used in biomedical research and empiric versus genomics-based therapy and **Chapter 2** *What's in a Gene?* – A discussion of the basics of DNA. |
| Week of April 9, 2007 | **Chapters 3 & 4** *The Human Genome* and *DNA Variation* –A discussion of the human genome project and the variations in the human genome, including SNPs mutations and haplotypes. |
| Week of April 23, 2007 | **Chapters 5 & 6** *Examining our Genes, Evaluating and Regulating Genetic Tests and Assessments* –Overview of genetic tests, what they can do and how they need to be assessed to ensure reliability and validity for patients. |
| Week of April 30, 2007 | **Clinical Trial Design I**<br>Text: *Understanding Clinical Trial Design: A Tutorial for Advocates (Research Advocacy Network, Perlmutter)* |
| Week of May 7, 2007 | **Clinical Trial Design II** |
| May 16, 17, 2007 | **Two-day intensive workshop** on genomics, proteomics, pharmacogenetics, and how advocates incorporate this knowledge in their research advocacy activities *See program |
| June 1- 5, 2007 | **ASCO Annual Meeting** in Chicago, Illinois (travel scholarships to attend will be included in the program participation) |
| June, July, 2007 | Development of a written **case study** applying genomics to research advocacy activities |
| July, 2007 | **Genomics Chapter 7**-*Ethical, Legal and Social Implications of Genomics* A discussion of the practical ethical concerns and questions related to genomics. |
| August, 2007 – December, 2007 | Identification of mentee(s)<br>Mentoring activities |
| August, 2007 – December, 2007 | **Advocate Grand Rounds** |

## Preliminary Program for Research Advocacy Mentor Workshop, Indianapolis, Indiana

*Speakers and locations are subject to change.*

| **May 15, 2007** | **University Place Hotel and Conference Center** | |
|---|---|---|
| 3:00 – 6:00 | Arrival | |
| 6:30- 8:30 | Welcome Reception and Mentoring Assessment | |
| | | |
| **May 16, 2007** | **University Place Hotel and Conference Center and Schneider Lab** | |
| 8:30 – 9:30 | How Genomics Has Changed Cancer Research | George Sledge, MD |
| 9:30 – 9:45 | Break | |
| 9:45- 10:45 | Working group - How genomics affects clinical trial design and research advocacy activities | Facilitated discussion |
| 10:45- 11:00 | Break | |
| 11:00 – 12:00 | Friends for Life Enrollment and Serum Collection | |
| 12:00 – 1:00 | Lunch | |
| 1:00 – 2:15 | How is proteomics incorporated into clinical trials? | Bob Hickey, PhD |
| 2:15 – 2:30 | Break | |
| 2:30 – 4:30 | Lab tour and activity using genomics (DNA isolation, etc.) | Bryan Schneider, MD |
| | | |
| 6:00 | Bus leaves for Dinner Location | |
| 6:30- 9:00 | Dinner | Location TBA |
| | | |
| **May 17, 2007** | **University Hospital Conference Room and Schneider Lab** | |
| 8:30 – 9:30 | Pharmacogenetics and how it has changed cancer research | Bryan Schneider, MD |
| 9:30 – 9:45 | Break | |
| 9:45- 10:45 | Tissue Banking-"Mary Ellen's Tissue Bank" | Anna M. Storniolo, MD |
| 10:45 – 11:00 | Break | |
| 11:00 – 12:00 | Working group - How pharmacogenetics is incorporated into research advocacy activities | |
| 12:00 -1:00 | Lunch | |
| 1:00 – 2:30 | Lab activity using pharmacogenetics | Bryan Schneider, MD |
| 2:30 – 2:45 | Break | |
| 2:45 – 3:30 | Mentoring exercise | |
| 3:30 – 4:30 | Case Study Development | |
| 4:30 | Adjourn | |

## Accomplishments

- Patient advocates participated in monthly core conference calls and meetings with Center staff.
- An Advocate Lecture Series was developed and offered. The series is available on the Research Advocacy Network and Hoosier Oncology Group web sites. Over 60 advocates registered for the three part series which covered the specific aims of the grant studies as well as scientific information on genomics, proteomics, and pharmacogenetics. Pathology and tissue collection was also covered.
- A newsletter was developed to provide general information to young breast cancer survivors about the Center. "In the Pink" was circulated by the IU center and is archived on their website.
- A Mentor Program for Research Advocates was planned and will be delivered in 2007.

## Conclusions

- Advocates and researchers can work together to help ensure the success of research studies.
- Developing a network of knowledgeable and interested advocates and advocate organizations can increase awareness and understanding of specific research projects.
- Providing advocates with education about the science of genomics through webinars can be replicated to reach not only a greater number of breast cancer advocates but patient advocates in other cancers.